AWARD NUMBER: W81XWH-14-1-0302

TITLE: A Translational Pathway Toward a Clinical Trial Using the Second-Generation AAV Micro-Dystrophin Vector

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REPORT DATE: September 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
September 2015	Annual	1 Sep 2014 - 31 Aug 2015

4. TITLE AND SUBTITLE **A Translational Pathway Toward a Clinical Trial Using the Second-Generation AAV Micro-Dystrophin Vector**

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-14-1-0302

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Missouri System
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MO 65211-3020

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Duchenne muscular dystrophy (DMD) is a life threatening disease affecting all muscles in the body. An important therapeutic goal of DMD gene therapy is to deliver a therapeutic gene to all muscles in the body. The overarching goal of this project is to achieve systemic AAV-8 mediated expression of a low immunogenic human micro-dystrophin gene to young adult affected dogs. In this funding period, we demonstrated that we can achieve efficient whole body skeletal muscle and heart gene transfer in neonatal dogs with AAV-8 by a single intravenous injection. We also showed that systemic delivery of a canine micro-dystrophin AAV vector is safe in young adult affected dogs. These results established a solid foundation to test systemic low-immunogenic human microgene delivery in young adult affected dogs in subsequent years of this project. In addition, we have performed a comprehensive review on the current status of DMD gene therapy in the canine model. We also contributed another article and reviewed the use of animal models in the development of DMD gene therapy.

15. SUBJECT TERMS
Duchenne muscular dystrophy, DMD, dystrophin, micro-dystrophin, adeno-associated virus, AAV, muscle, gene therapy, systemic gene delivery, canine model

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	16	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Abstract

Duchenne muscular dystrophy (DMD) is a life threatening disease affecting all muscles in the body. An important therapeutic goal of DMD gene therapy is to deliver a therapeutic gene to all muscles in the body. The overarching goal of this project is to achieve systemic AAV-8 mediated expression of a low immunogenic human micro-dystrophin gene to young adult affected dogs. In this funding period, we have demonstrated that we can achieve efficient whole body skeletal muscle and heart gene transfer in neonatal dogs with AAV-8 by a single intravenous injection. We have also showed that systemic delivery of a canine micro-dystrophin AAV vector is safe in young adult affected dogs. These results established a solid foundation to test systemic low-immunogenic human microgene delivery in young adult affected dogs in subsequent years of this project. In addition, we have performed a comprehensive review on the current status of DMD gene therapy in the canine model. We also contributed another article and reviewed the use of animal models in the development of DMD gene therapy.

1. Introduction

Duchenne muscular dystrophy (DMD) is a life threatening disease affecting approximately one in 5,000 newborn boys. It is caused by dystrophin deficiency. Currently there is no effective therapy to target dystrophin deficiency in this lethal disease. Adeno-associated virus (AAV)-mediated micro-dystrophin gene therapy has resulted in unprecedented successful in treated mouse models of DMD. We have recently shown that AAV micro-dystrophin gene therapy can also reduce muscle disease in dystrophin-deficient DMD dogs following direct local muscle injection. Since DMD affects all muscles in the body, we propose here to develop systemic AAV-8 human micro-dystrophin gene therapy in the canine model.

2. Keywords

Duchenne muscular dystrophy, DMD, dystrophin, micro-dystrophin, adeno-associated virus, AAV, muscle, gene therapy, systemic gene delivery, canine model

3. Accomplishments

Major goal. We have two specific aims. Our first aim is to design and validate a new, low immunogenic human ΔR2-15/ΔR18-19/ΔR20-23/ΔC microgene AAV vector in adult dystrophic dogs by direct local injection. Our second aim is to test systemic gene therapy in young adult DMD dogs with the newly developed microgene AAV vector.

Accomplishments.

Aim 1. Our initial discovery that AAV microgene vector can induce immune reaction in affected dogs was made with a canine microgene AAV vector called SJ13 (Shin et al., 2013). As the first step towards translation to a human microgene vector, we tested if we can reduce immunogenicity with our original canine SJ13. To further enhance the function of the micro-dystrophin gene, we first engineered the dystrophin syntrophin/dystrobrevin binding site (syn/dbr) into SJ46 and the resulting

construct was termed YL391. Next, we added the proposed hematopoietic lineage specific microRNA 142-3 binding site (mirT) into YL391. The resulting vector YL396 has a size of 5,124bp. Next we replaced the CMV promoter with the muscle specific Spc5-12 promoter and generated the vector YL398. Since YL398 has a size (4,971bp) close to that of AAV packing limit (5,000bp). So we generated another vector called YL397 (4,849bp). **Figure 1** showed cloning steps of these vectors.

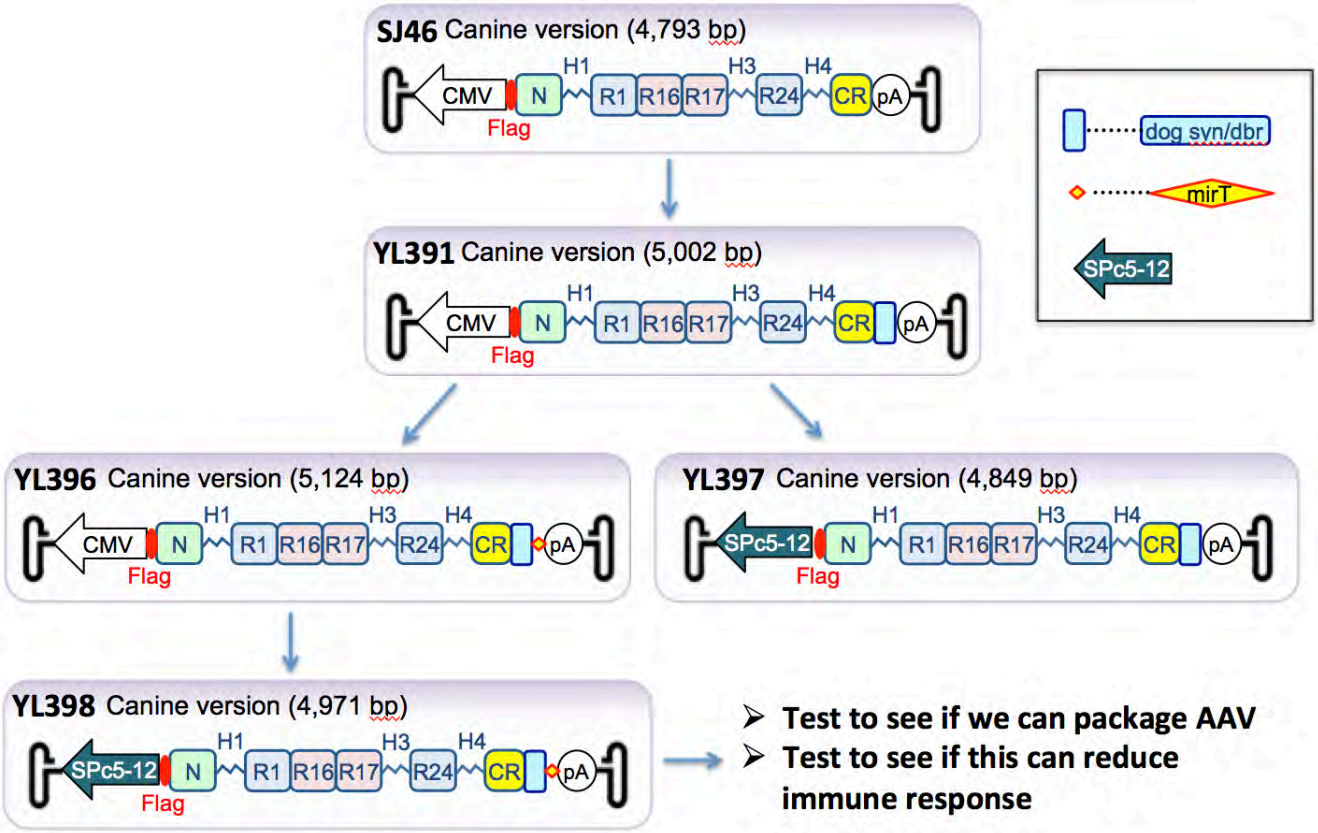

Figure 1. Engineering of the original canine microgene vector SJ46 to test the concept of reduced immunogenicity by the muscle specific promoter and miR142-3p binding site.

Next, we tested whether we can successfully package YL398 into a functional AAV vector and whether the presumed low-immunogenic property of YL398 can indeed reduce immunogenicity. We successfully achieved high titer YL398 AAV virus. Injection in one adult affected dog revealed great reduction of CD4+ and CD8+ T cell infiltration (Figure 2).

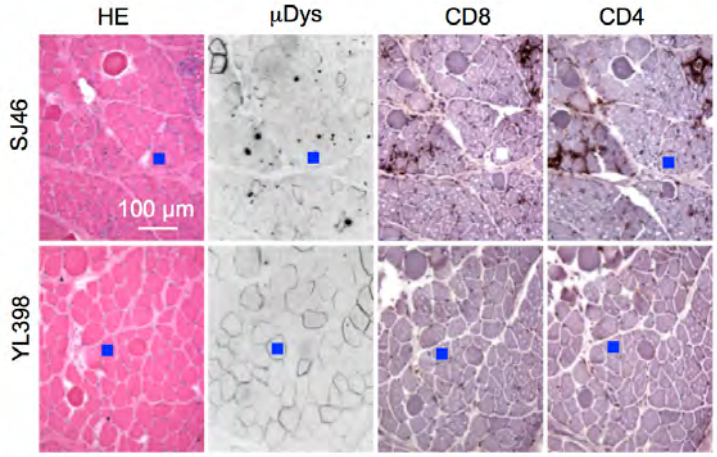

Figure 2. The use of muscle specific promoter and inclusion of hematopoietic lineage microRNA 142-3p target site in the AAV vector greatly reduced T cell infiltration. This preliminary data confirmed our original hypothesis and provided a strong rational to engineer novel human microgene vector.

Together, we have successfully demonstrated that our originally proposed strategy is a valid approach to reduce immunogenicity of the AAV vector in dystrophic dogs.

To engineer the human version of the low-immunogenic AAV micro-dystrophin vector, we first replaced the dog microgene with a codon-optimized human microgene and generated construct XP8. This packaging plasmid of this construct has a backbone of ~3.6kb. This is smaller than the AAV packaging limit. Hence, there is a high possibility that the backbone can be packaged during vector production. Since the backbone is composed of the bacterial replication origin and the bacterial ampicillin resistant gene, there will be highly immunogenic in human patients. Thus, we further modified XP8 with enlarged backbone. We made two different versions of backbone enlarged XP8 and named them as XP9 and XP11 (Figure 3).

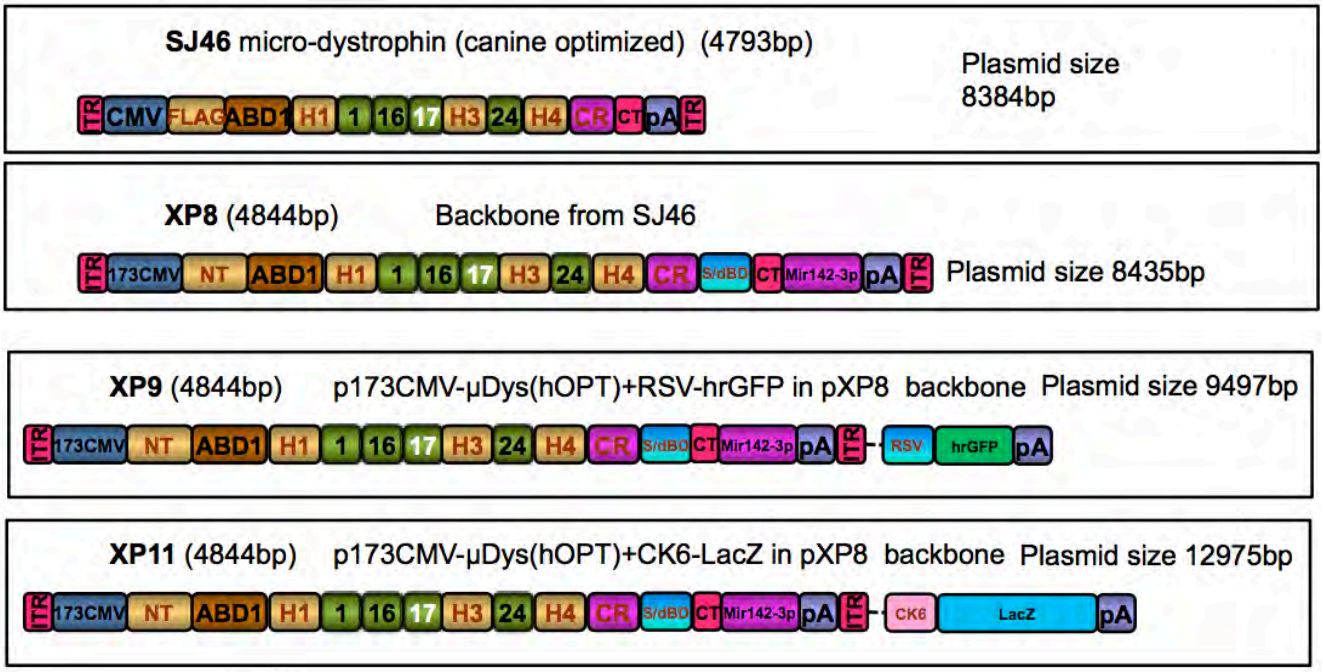

Figure 3. Cartoon outline of the construct SJ46 (the original canine microgene vector) and three human microgene vectors including XP8, XP9 and XP11.

During our engineering of the new canine and human gene vectors, a new study was published suggesting that the CpG motif is another critical determining factor for AAV-induced immune response (Faust et al., 2013). In light of this new finding, we decide to upgrade our newly generated human microgene vector by removing the CpG motif (see Plan for the next reporting period).

Aim 2. Our eventual goal is to test systemic AAV-8 delivery in young adult dogs with our low-immunogenic vector. As a first step toward this goal, we tested if AAV-8 can efficiently transduce newborn dogs after systemic delivery (Pan et al., 2015). We used newborn dogs because they are minimally immunogenic so that we can test if AAV-8 can efficient transduce dog muscles before we clone our new CpG-free vector. The experimental protocol and dogs are summarized in **Table 1**.

As safety is the most important concern, we first looked the blood panel in treated dogs. Although a few values are slightly over the range of our un-injected controls, they either reflect transgene expression (alkaline phophotase) or are not clinically meaningful (**Table 2**).

TABLE 1. SUMMARY OF EXPERIMENTAL PROTOCOL AND DOGS

	Christa	*Barbara*	*Artemis*[a]	*Dojo*[a]	*Generic*
Gender	Female	Female	Female	Male	Female
Genotype	Carrier	Carrier	Carrier	Normal	Carrier
Age at the time of injection (day)	2	2	2	2	N/A
BW at the time of injection (g)	380	380	412	520	N/A
AAV serotype	AAV-9	AAV-9	AAV-8	AAV-9	N/A
Injection volume (ml/kg BW)	9.21	14.47	14.56	8	N/A
AAV dosage (vg particle/kg BW)	6.14×10^{14}	9.65×10^{14}	9.06×10^{14}	2×10^{14}	N/A
BW at 2.5 months of age (kg)	5.40	5.44	6.02	—	5.8
Age at necropsy (month)	2.5	2.5	2.5	6	2.5

AAV, adeno-associated virus; BW, body weight; N/A, not applicable; —, not available.
[a]Dogs used in previous published experiment.

TABLE 2. BLOOD EXAMINATION RESULTS

	Age (2.5 months)			*Age (1.5–3.5 months)* (n=20)	
	Christa	*Barbara*	*Artemis*	*Uninjected control, mean ± SEM*	*Uninjected control, range*
Calcium (mg/dl)	11.8	11.8	10.8	11.2 ± 0.1	10.5–11.9
Chloride (mEq/liter)	109	**113**[a]	109	106.6 ± 0.6	99–111
Phosphorus (mg/dl)	9.4	9.2	9.2	8.5 ± 0.2	7.0–10.1
Potassium (mEq/liter)	**7**	5.1	5	5.8 ± 0.1	5.2–7.1
Sodium (mEq/liter)	142	145	148	141.5 ± 0.8	133–148
Albumin (g/dl)	2.5	2.6	2.3	2.7 ± 0.1	2.1–3.6
Alkaline phosphatase (ALP) (U/liter)	**209**	**310**	**239**	147.9 ± 5.4	111–200
Alanine aminotransferase (ALT) (U/liter)	70	52	42	25.7 ± 4.4	9–97
ALP/ALT ratio	2.9	5.9	5.7	8.1 ± 1.1	1.3–22
Anion gap (mEq/liter)	20	**17**	25	22.2 ± 0.5	19–28
Cholesterol (mg/dl)	236	194	186	254.2 ± 14.7	173–427
Creatinine (mg/dl)	0.5	0.4	0.5	0.4 ± 0.0	0.2–0.7
Gamma-glutamyl transpeptidase (GGT) (U/liter)	<3	2	<3	0.9 ± 0.4	0–6
Globulin (g/dl)	2.2	2.2	1.9	2.2 ± 0.1	1.7–2.7
Glucose (mg/dl)	**197**	110	**88**	105.3 ± 2.4	89–126
Total bilirubin (mg/dl)	0.2	0.2	0.3	0.2 ± 0.0	0.1–0.4
Total CO_2 (mEq/liter)	20	20	19	18.6 ± 0.9	7–24
Total protein (g/dl)	4.7	4.8	4.2	4.9 ± 0.1	4.1–6.3
Urea nitrogen (mg/dl)	10	14	19	11.8 ± 1.4	4–22

[a]Bold font indicates the value is not within the range of uninjected control.

In our previous study, we demonstrated bodywide skeletal muscle transduction after intravenous injection of 2 x 10^{14} vg /kg of AAV-9 in newborn puppies. However, gene transfer in many muscles (such as RF, CS, and AR) remains sub-optimal (50-80%) (**Figure 4A**). A 3-fold increase in the vector dose (to 6.14 x 10^{14} vg /kg) resulted in complete (~ 100%) transduction of every limb muscle in Christa (**Figure 4A**). Boosting the dose further to 9.65 x 10^{14} vg /kg in Barbara (~ 5-fold higher than the dose used in Dojo) increased the vector genome copy number in most muscles but it did not yield dramatically much higher AP activity in muscle lysate suggesting a saturation effect in skeletal muscle (**Figure 4C, D**).

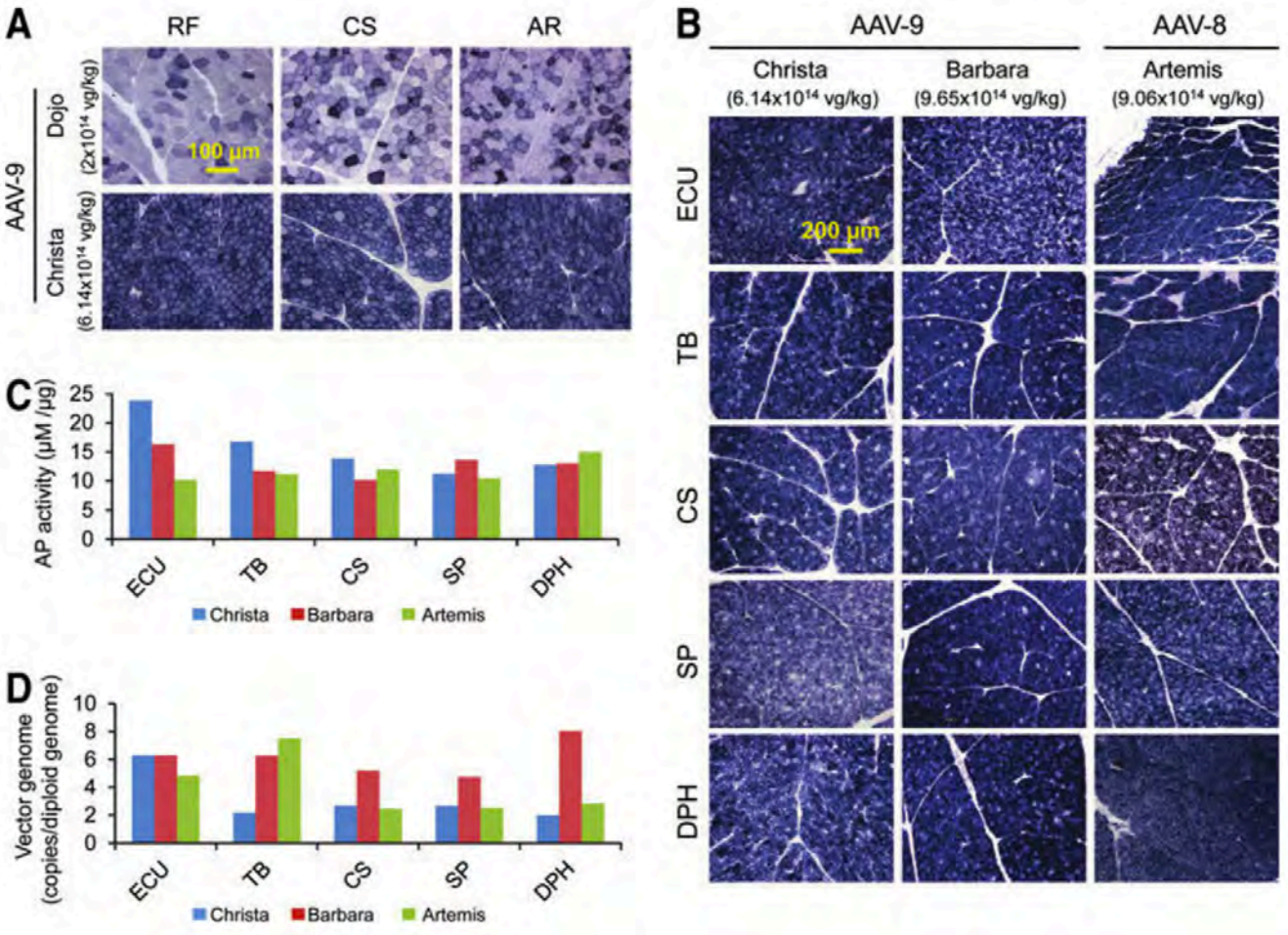

Figure 4. Intravenous injection of high dose AAV-9 results in improved skeletal muscle transduction in neonatal dogs. A, Representatively photomicrographs of AP histochemical staining from muscles that were injected with low (Dojo, 2 x 10^{14} vg /kg) and medium (Christa, 6.14 x 10^{14} vg /kg) doses of the AAV-9 AP reporter vector. RF, rectus femoris; CS, cranial sartorius; AR, abdominal rectus. **B,** Representatively photomicrographs of skeletal muscle AP histochemical staining from AAV-9 injected dog Christa and Barbara as well as AAV-8 injected dog Artemis. ECU, extensor carpi ulnaris; TB, triceps brachii; CS, cranial sartorius; SP, superficial pectoralis; DPH, diaphragm. **C,** Quantitative examination of AP activity in muscle lysate. **D,** Comparison of the AAV vector genome copy number in different skeletal muscles.

Next, we examined AAV transduction in the heart. In our published studies with AAV-9 (1 to 2.5 x 10^{14} vg /kg), hardly any expression was detected in the heart. In Christa (6.14 x 10^{14} vg /kg), AP positive cells became readily detectable by histochemical staining in the interventricular septum, left ventricle and papillary muscle (**Figure 5A**). A dose-dependent increase in AP expression was observed in almost every region of the heart in Barbara (9.65 x 10^{14} vg /kg). Widespread AP positive cells were seen in the left atrium, interventricular septum, papillary muscle and left ventricle (**Figure 5A**). However, it never reached the level seen in Artemis, a puppy injected with 9.06 x 10^{14} vg /kg of AAV-8 (**Figure 5A, B**). Interestingly, on histochemical staining, the right heart (RA and RV) was efficiently transduced by AAV-8 but barely transduced by AAV-9 (**Figure 5A**).

AAV copy number quantification revealed an interesting pattern. In the right ventricle and interventricular septum, we detected more vector genome in AAV-8 injected Artemis, consistent with high transduction. But in the right atrium, similar number of the AAV genome copy was found despite a substantially much more robust expression in the AAV-8 injected puppy (**Figure 5**). Most surprisingly, in the left atrium, papillary muscle and left ventricle, AAV copy number in Barbara (9.65 x 10^{14} vg /kg of AAV-9) was 3 to 5-fold higher than that of Artemis (9.06 x 10^{14} vg /kg of AAV-8). However, the high copy number did not result in high expression suggesting that a significant portion of the AAV-9 genome may have been trapped in a yet to be defined dead compartment and/or not converted to the transcription-competent form in cardiomyocytes and cannot express the transgene.

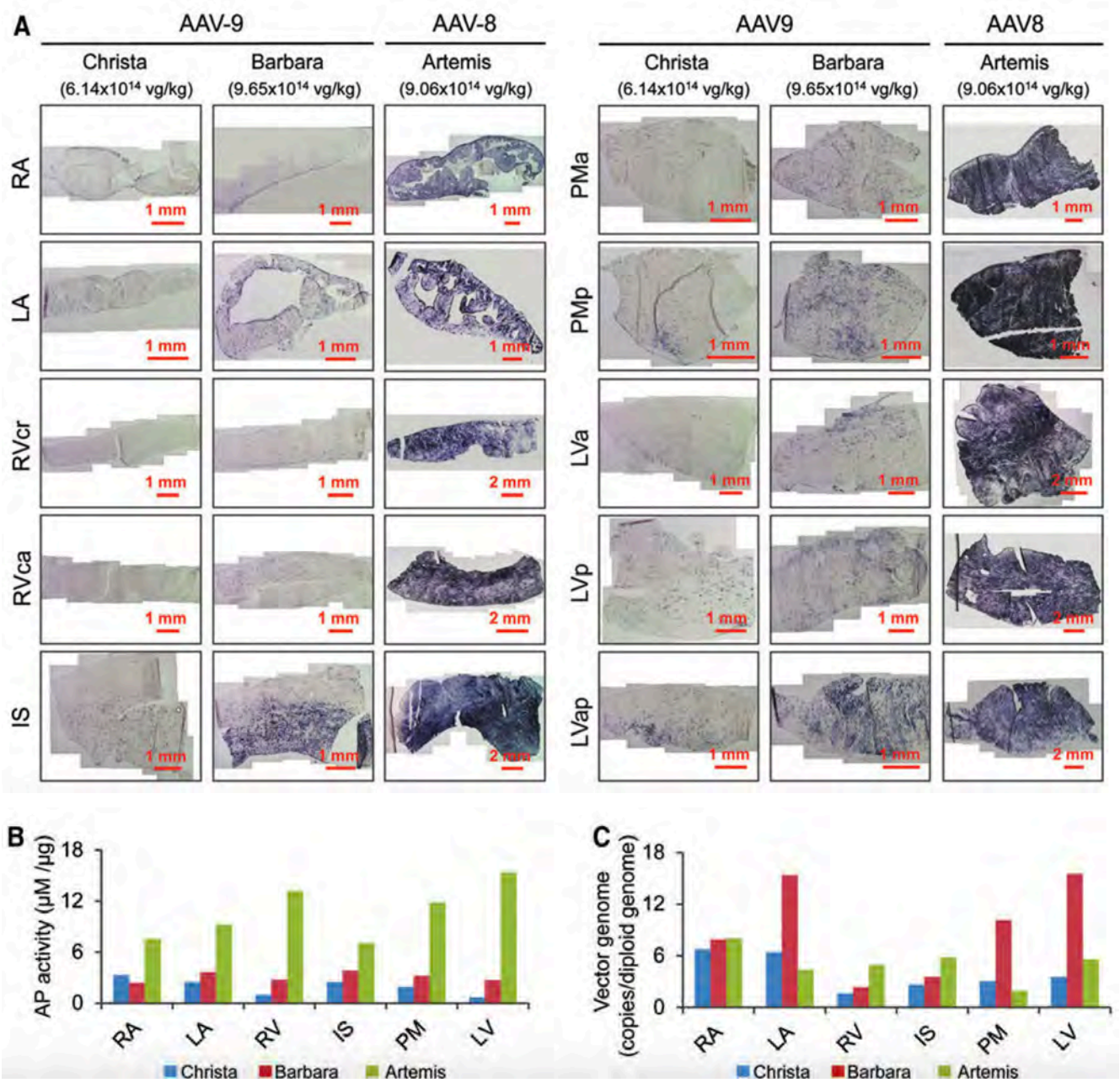

Figure 5. AAV-8 is more efficient than AAV-9 in transducing neonatal dog heart. A, Representative lower magnification microphotographs of AP staining in different parts of the heart including from right atrium (RA), left atrium (LA), right ventricular cranial wall (RVcr), right ventricular caudal wall (RVca), interventricular septum (IS), anterior papillary muscle (PMa), posterior papillary muscle (PMp), left ventricular anterior wall (LVa), left ventricular posterior wall (LVp) and left ventricular apex (LVap). B, Quantitative examination of AP activity in muscle lysate from different parts of the heart. C, AAV genome copy quantification in different parts of the heart. RA, right atrium; LA, left atrium; RV, right ventricle; IS, interventricular septum; PM, papillary muscle; LV, left ventricle.

In summary, our data suggests that AAV-8 can lead to bodywide muscle transduction (Pan et al., 2015). Importantly, we found AAV-8 can transduce newborn dog heart at high efficiency independent of the vector dose (Pan et al., 2015). Because cardiomyopathy is a major cause of death in DMD patients, this piece of new data provides further support to develop AAV-8 microgene therapy in the canine model.

A previous study suggests that systemic AAV delivery in affected dogs may induce an inflammatory response and further worsen the disease (Kornegay et al., 2010). To probe the feasibility and safety of systemic AAV gene therapy in young adult DMD dogs, we performed a study using our previously published AAV vectors including a alkaline phosphatase reporter gene vector and a micro-dystrophin gene vector (Yue et al., 2015). Experimental dogs are listed in **Table 3** next to this paragraph.

Dog name	Bouchelle	Stephan	Brooke
Dystrophin gene mutation	Intron 19 insertion	Intron 6 point mutation	Intron 13 insertion
Gender	Male	Female	Male
Body weight at injection (kg)	3.7	3.5	3.2
Body weight at necropsy (kg)	10.6	10.6	11.3
Promoter	RSV	CMV	CMV
Transgene	Human AP reporter gene	Canine μ-dystrophin	Canine μ-dystrophin
Total AAV injected (vg particles)	7.09×10^{14}	1.77×10^{15}	2.0×10^{15}
Vector dose (vg particles/kg BW)	1.92×10^{14}	5.04×10^{14}	6.24×10^{14}
Vector volume (ml/kg BW)	4.9	5.7	6.2
Age at AAV injection (month)	2.0	1.8	1.8
Age at biopsy (month)	3.0	2.8	2.8
Age at necropsy (month)	5.5	5.3	5.8

Dog Bouchelle was injected with 1.92 x 10^{14} vg particles/kg (7.09 x 10^{14} vg particles total) of a Rous sarcoma virus (RSV) promoter driven alkaline phosphatase (AP) reporter AAV vector (Table 3). This dog also received five-week transient immune suppression. We also delivered a micro-dystrophin AAV vector to affected dogs Stephan (5.04 x 10^{14} vg particles/kg, 1.77 x 10^{15} vg particles total) and Brooke (6.24 x 10^{14} vg particles/kg, 2.00 x 10^{15} vg particles total) (Table 3).

We first examined the potential toxicity. We found that the body weights of all these three injected dogs were within the range of untreated affected dogs in our colony (Figure 6). On the blood chemistry panel, we did not find any abnormality (Figure 7).

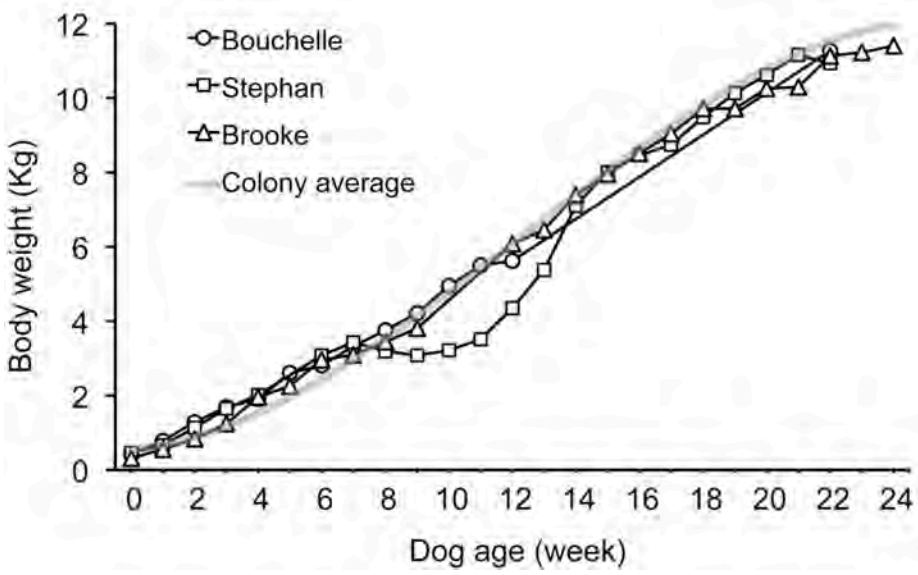

Figure 6. Body weight changes over 24 weeks in experimental dogs.

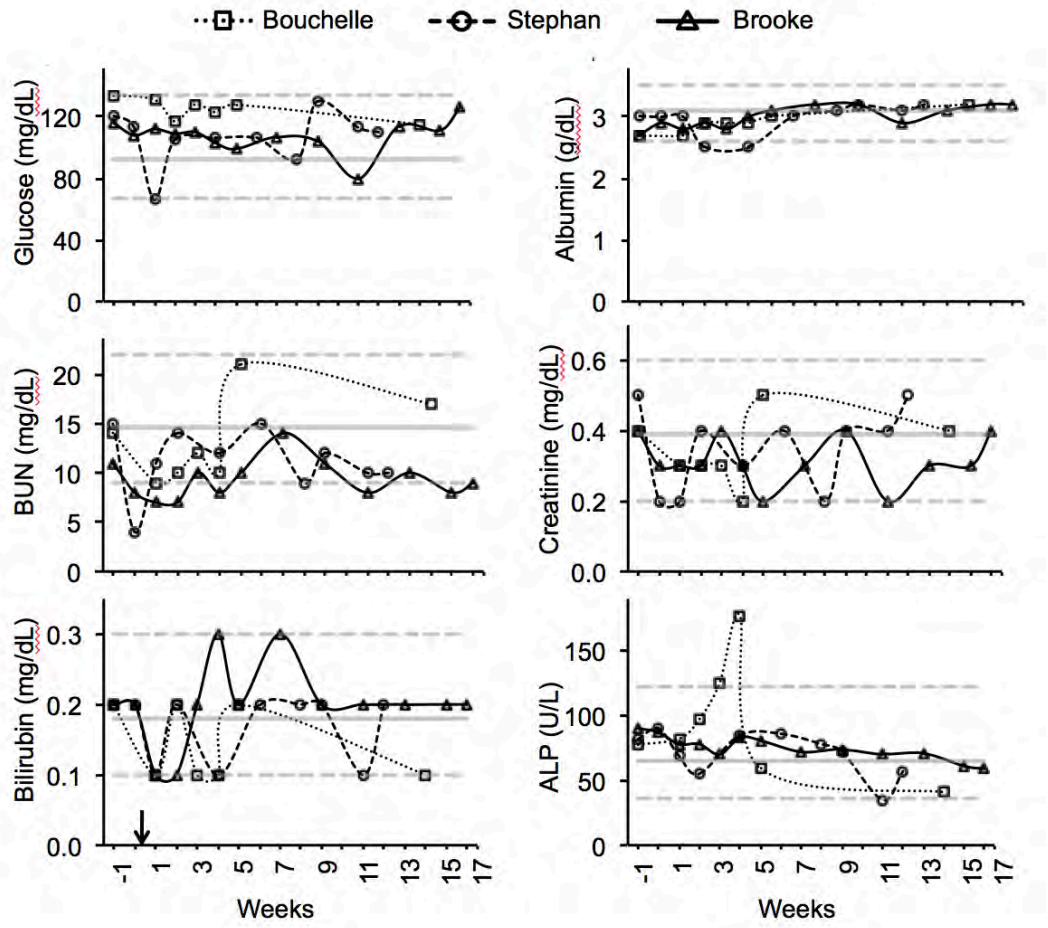

Figure 7. Blood biochemistry in experimental dogs. Dotted gray lines, the maximal and minimal values for age-matched untreated DMD dogs in our colony (N = 31). Solid gray line, the average value of age-matched untreated DMD dogs in our colony (N = 31).

Since our ultimate goal is to see if we can achieve bodywide systemic delivery to all muscles in the affected dog. We evaluated transgene expression and vector genome copy number. Figure 8 shows the results from the dog Bouchelle. Figure 9 shows the results from dogs Stephan and Brooke.

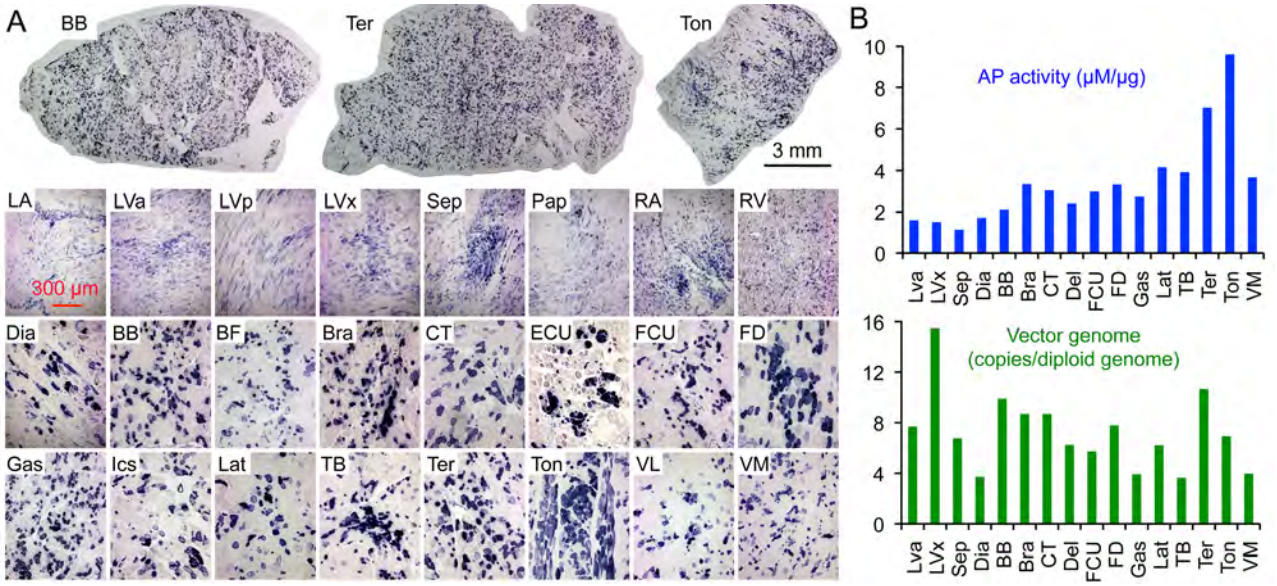

Figure 8. A single intravenous injection resulted in robust bodywide transduction with a reporter AAV vector.

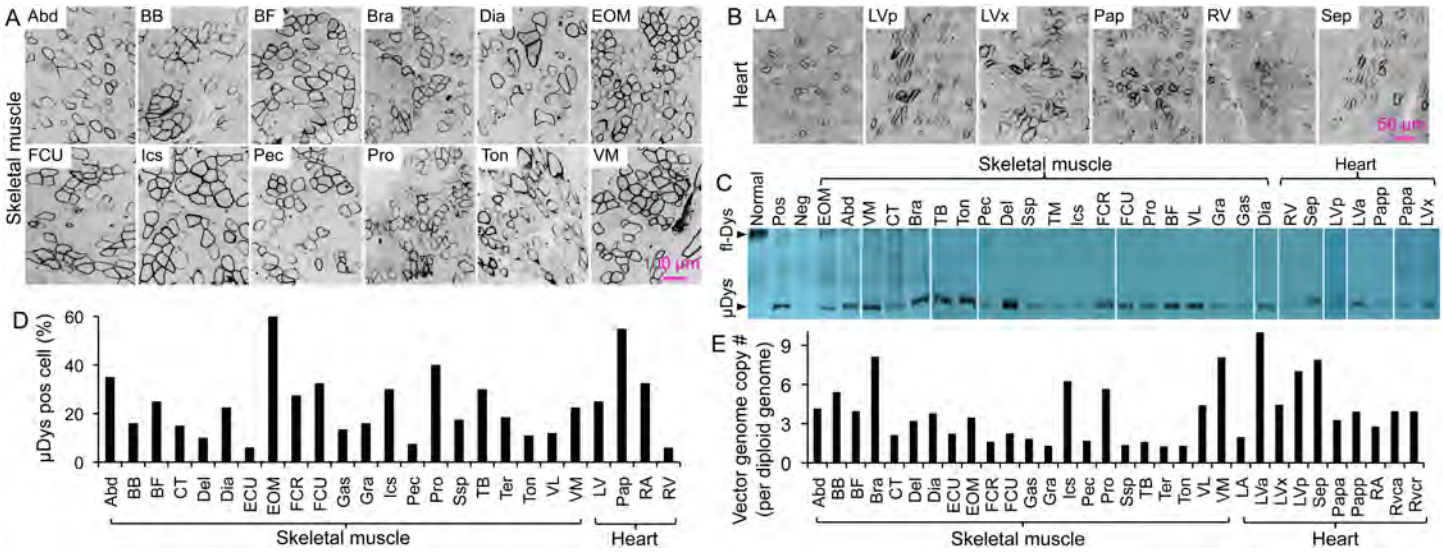

Figure 9. A single intravenous injection resulted in robust bodywide transduction with a micro-dystrophin AAV vector

In summary, our results suggest that systemic AAV gene transfer is safe and efficient in dystrophic large mammals (Yue et al., 2015). Our data established a strong foundation to test systemic AAV gene therapy in the canine model using the CpG-free human microgene vector.

Accomplishments related to both Aims 1 and 2. Because our goal is to develop AAV micro-dystrophin gene therapy on animal models, so we conducted a comprehensive review on the current status of the animal models used in DMD gene therapy (with specific emphasis on the canine model) (McGreevy et al., 2015). We further reviewed the current status of DMD gene therapy in the canine model (Duan, 2015). In addition, we further characterized the clinical phenotype in the DMD dogs that we have proposed to use in this project (Duan et al., 2015). Specifically, we quantified the loss of ambulation by 6 months of age in our colony and several other colonies. This study suggests that the early loss of ambulation is not a typical clinical presentation in affected dogs (Table 4).

Table 4. Complete loss of ambulation is not a clinical feature in young adult DMD dogs.

Investigator (or reference paper)	Colony location	Strain background	Mutation	Sample size	Loss of ambulation by 6 months	
					Number	Percentage (%)
Carlos Ambrosio	Brazil	Golden retriever	Intron 6 point mutation (GRMD)	160	1	0.63
Dongsheng Duan	Columbia, MO	Golden retriever	Intron 6 point mutation (GRMD)	130	0	0.00
		Corgi	Intron 13 insertion			
		Labrador	Intron 19 insertion			
		Hybrid	Mixed			
Bruce Smith	Auburn, AL	Corgi	Intron 13 insertion	30	0	0.00
		Labrador	Intron 19 insertion			
		Labradoodle	Unknown			
		Springer	Unknown			
Lee Sweeney	Philadelphia, PA	Golden retriever	Intron 6 point mutation (GRMD)	35	0	0.00
Valentine et al., 1988	Ithaca, NY	Golden retriever	Intron 6 point mutation (GRMD)	25	0	0.00
		Golden retriever/ Beagle hybrid	Intron 6 point mutation (GRMD)			
Total				**380**	**1**	**0.26**

Training and professional development opportunities. Nothing to report.

Dissemination of the results. All above mentioned studies and review articles have been published in peer-reviewed scientific journals.

Plan for the next reporting period.

Aim 1. We initially proposed to reduce AAV micro-dystrophin vector immunity by (1) removing the immunogenic Flag tag, (2) replacing the ubiquitous CMV promoter with the muscle specific SPc5-12 promoter, and (3) including the miR142-3p binding site. However, after the submission of our original application, a study was published by Faust et al suggesting that the CpG motif in AAV vector may represent another critical determining factor for AAV induced immune response (Faust et al., 2013). To develop a vector that has minimal possibility to induce the immune response in human DMD patients, we decided to remove all CpG motifs in our proposed AAV micro-dystrophin vector. We calculated the number of the CpG motif in our proposed vector. There are 29 CpG in the SPc5-12 promoter, 32 CpG in the inverted terminal repeats (ITRs) and 193 CpG in codon-optimized human micro-dystrophin gene.

The promoter specificity and activity is determined by the nucleotide sequence. It is not clear whether elimination of the CpG motif can compromise the specificity and activity of the SPc5-12 promoter. To this end, we have decided to (1) remove CpG motifs in SPc5-12 promoter and test

whether the CpG-free SPc5-12 promoter can still maintain strong activity in myoblasts in vitro and in mouse muscle tissue in vivo; (2) explore alternative muscle specific promoters to see if these promoters can be engineered into CpG-free promoters. Currently, our top candidate is the CK8 promoter developed by Drs Steve Hauschka and Jeff Chamberlain's group; and (3) synthesize a CpG-free muscle-specific promoter de novo. We are currently exploring all these options.

The ITRs are the only viral components in the AAV vector. They flank the expression cassette and serve as the packaging and viral replication signal. Similar to the modification of the promoter, elimination of the CpG motif in an AAV vector also have a set of risks. For example it may compromise vector production (such as failure to package) and gene transfer efficiency. To this end, we have decided to generate the CpG-free ITR and compare it with the original CpG-containing ITR. We are currently in the process of cloning these vectors. After we get these vectors, we will quantify the viral yield to see if CpG-depletion affects AAV production. We will also test the transduction efficiency of the new vector in mdx mice to see if it can yield similar transduction efficiency as the original vector.

To remove CpG motifs in the human micro-dystrophin gene, we will synthesize new CpG-free human micro-dystrophin. We will then compare the immunogenicity and function of the CpG-free microgene with the original CpG-containing microgene in the context of the AAV vector in mdx mice.

Aim 2. We will start testing systemic transduction of AAV-8 in young adult affected dogs using our existing AAV constructs. To minimize immune reaction, we will apply transient immune suppression using a protocol we recently published. This study will clear the path to the eventual test of our final CpG-free human micro-dystrophin vector in year 3.

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4. Impact. Nothing to report.

5. Changes/Problems.

Changes in approach and reasons for change.

During our engineering of the human micro-dystrophin gene vector, a new study was published suggesting that the CpG motif is another critical determining factor for AAV-induced immune response (Faust et al., 2013). In our previous design, we have not considered the CpG motif. In light of this new finding by Faust et al, we decide to upgrade our human microgene vector by removing the CpG motif. Specifically, we plan to remove the CpG from the entire expression cassette and AAV-ITR to further improve the safety of the human micro-dystrophin vector.

Actual or anticipated problems or delays and actions or plans to resolve them.

A CpG-free micro-dystrophin gene has never been tested. It is possible that such changes may introduce splicing signals and/or other untoward motifs in the microgene. In the worst-case scenario, we may not get a functional micro-dystrophin protein expressed from the CpG-free construct. To this end, we will test the CpG-free microgene in our existing expression cassette first to assure the function.

Currently, there is not a CpG-free muscle specific promoter. We have proposed several strategies to generate a CpG-free muscle specific promoter. If this does not work, we will use a muscle-specific promoter with the fewest number of the CpG motifs.

Since the ITR is the AAV replication original and packaging signal. Any engineering of the ITR may lead to untoward consequences on vector production and vector activity. If this becomes an issue, we will use the original ITR in our construct. Based on Faust et al study (Faust et al., 2013), removing CpG from the expression cassette alone still offers immunological advantages.

Changes that had a significant impact on expenditures.

Nothing to report.

Significant changes in vertebrate animals.

Nothing to report.

6. Products

Peer-reviewed publications

1) McGreevy JW, Hakim CH, McIntosh M, **Duan D.** *Animal models for Duchenne muscular dystrophy: from basic mechanisms to gene therapy.* **Disease Model and Mechanism** 8(3):195-213, **2015**.

2) Pan X, Yue Y, Zhang K, Hakim CH, Kodippili K, McDonald T, **Duan D.** *AAV-8 is more efficient than AAV-9 in transducing neonatal dog heart.* **Human Gene Therapy Methods**. 26(4):54-61, **2015**.

3) **Duan D**. *Duchenne muscular dystrophy gene therapy in the canine model.* **Human Gene Therapy Clinical Development**. 26(3):157-169, **2015**.

4) **Duan D**, Hakim CH, Ambrosio C, Smith B, Sweeney L. *Early loss of ambulation is not a representative clinical feature in Duchenne muscular dystrophy dogs.* **Disease Model and Mechanism** 8(3):193-194, **2015**.

5) Yue Y, Pan X, Hakim CH, Kodippili K, Zhang K, Shin J-H, Yang HS, McDonald T, **Duan D.** *Safe and bodywide muscle transduction in young adult Duchenne muscular dystrophy dogs with adeno-associated virus.* **Human Molecular Genetics**. 24(20):5880-5890, **2015**. (highlighted in *Human Gene Therapy Clinical Development* 26(4):213-214, 2015)